Annals
of the
Missouri Botanical Garden

Volume 54 1967 Number 2

THE BIOTIC RELATIONSHIP OF ANTHOCEROS AND PHAEOCEROS TO CERTAIN CYANOPHYTA[1]

BY JOHN E. RIDGWAY[2]

Department of Botany, Washington University, St. Louis, Missouri

ABSTRACT

When the *Nostoc* isolates entered into thalloid cavities of *Anthoceros* and *Phaeoceros* and produced the typical globose endophytic colonies and/or when the algal isolate grew upon the nitrogen-free substrate in intimate contact with the thalli, chlorosis in the experimental cultures was not present to the degree that it occurred in the control cultures. These observations suggest the ability of these blue-green algal isolates to fix atmospheric nitrogen and to provide an available source to the liverworts used in these experiments. Cytochemical and ultrastructural observations further suggest that the algal endophyte may also benefit from this relationship if the algae are able to catabolize carbohydrate components of the mucilaginous thalloid cavity.

The presence of blue-green algae in thalli of *Anthoceros* was first reported by Hofmeister (1862) who thought the dark, blue-green, roundish masses in the thallus to be gemmae. However, careful investigations by Leitgeb (1881) showed that they were *Nostoc* colonies within the mucilaginous *Anthoceros* cavities. He reported that the endophytism is effected by the movement of free *Nostoc* hormogonia through slit-like openings into the mucilage cavities. Once within the cavities the hormogonia grow into globular colonies. The presence of *Nostoc* within the thallus causes an increased, attenuated growth of the cells surrounding the cavity. According to Campbell (1918) and Parihar (1961) the "host" cells produce tubular filaments which ramify through the algal mass and become so interwoven with it that sections appear as loose parenchyma with *Nostoc* filaments occupying the interstices. The writer can confirm their observations.

Pierce (1906) reported that the presence of the endophytic blue-green alga was not necessary for growth of the gametophyte, and that in fact thalli grown

---

[1]Rewritten portion of a thesis submitted in partial fulfillment for the Doctor of Philosophy degree at the University of Texas.

[2]I wish to express appreciation to Professor H. C. Bold for his assistance and to the National Science Foundation (Grant No GB-313).

ANN. MISSOURI BOT. GARD. **54**(2): 95-102, 1967.

The previous issue of the ANNALS OF THE MISSOURI BOTANICAL GARDEN, Vol. **54**, No. 1, pp. 1-94, was published on June 12, 1967.

on sterilized soil without *Nostoc* grew better than those containing the alga. He further commented that there was no conceivable advantage in the enlargement of the mucilage chamber cells.

Since that time several investigators have isolated *Nostoc* from other sources—root parenchyma of *Gunnera* (Winter, 1934), roots of cycads, thalli of lichens, and the liverwort *Blasia* (Bond & Scott, 1955; Watanabe & Kiyohara, 1963)—and have been able to show through the use of isotopic nitrogen (N^{15}) the ability of the isolated algae to fix free nitrogen. None, however, has shown nitrogen fixation by a blue-green alga isolated from thalli of *Anthoceros*.

The purposes of this investigation were several: (1) to obtain axenic cultures of the blue-green algae isolated from thalli of *Anthoceros* spp. and *Phaeoceros* spp.; (2) to obtain and grow the thalli in axenic culture; (3) to attempt inoculation of the algal isolates into the thalli in culture utilizing a nitrogen-free medium; (4) to attempt inoculation of thalli with other blue-green algal isolates from other plants; and (5) to investigate the nature and specificity of the blue-green alga-liverwort relationship.

Materials and Methods

In June, 1961, collections of *Phaeoceros laevis* subsp. *carolinianus* (Michx.) Prosk. were made in Bastrop County, Texas. Additional specimens of this subspecies were collected in Jefferson County, Alabama, by T. E. Deason in the fall of 1962. Clonal, axenic cultures of these two collections were obtained by following the procedures of Näf (1958) as modified by Kelley & Postlethwait (1962). Two species of *Anthoceros* (H 13 *A. husnoti* Steph. and H. 166 *A. punctatus* L.) were obtained from the culture collection at the Czechoslavakian Academy of Sciences in Prague. These two living specimens were not axenic.

Of the blue-green algal isolates used in the experiment, seven strains were obtained from the blue-green algal collection maintained at the Czechoslavakian Academy of Sciences; two were supplied through the courtesy of Dr. Jack Meyers, and four were isolated from the collection of *Phaeoceros leavis* subsp. *carolinianus*, purified, and placed into axenic cultures in cooperation with Dr. Larry W. Jones. Collection and isolation data for all blue-green algal isolates used in the experiments are summarized in Table 1. The algal isolates were grown in 15 cm test tubes into which 10 ml of Bold's Basil Medium (BBM), lacking nitrogen, had been added (Bischoff & Bold, 1963). Transfers were made to new media every three weeks.

For the experiment sterile Petri dishes were divided into quadrants and partially filled with sterilized BBM nitrogen-deficient agar medium. Into the center of each quadrant a fragment (which contained at least one growing apex) of each of the four species of *Anthoceros* and *Phaeoceros* was transferred and carefully marked. To the surface of each of the four thalli per plate, three drops of a suspension of a given blue-green algal isolate were added. The preparations were then placed in an incubator in 250 ft c illumination at 15° with 12 hr diurnal light, and grown for 125 days.

Table 1. Collection data of the blue-green algal isolates.

Designation	Species	Isolator	Source
122*	*Anabaena variabilis*	Greifswald	unknown
123*	*Nostoc linckia*	unknown	unknown
373*	*Anabaena cylindrica*	Foog	unknown
388*	*Nostoc* sp.	Lhotský	*Blasia pusilla*
391*	*Nostoc* sp.	Lhotský	*Cycas circinalis*
393*	*Nostoc* sp.	Lhotský	*Encephalartos altensteinii*
395*	*Nostoc* sp.	Lhotský	*Gunnera chilensis*
396*	Nostoc sp.	Lhotský	*Stangeria paradoxa*
NMA	*Nostoc muscorum* A	unknown	unknown
NMB	*Nostoc muscorum* B	unknown	unknown
49	*Nostoc* sp.	Jones and Ridgway	*Phaeoceros laevis* (C. Texas)
50	*Nostoc* sp.	Jones and Ridgway	*Phaeoceros laevis* (W. Texas)
ND_1	*Nostoc* sp.	Jones	*Dion*
ND_2	*Nostoc* sp.	Jones	*Dion*

* Cultures obtained from the culture collection, Czechoslavakian Academy of Sciences, Prague.

Portions of thalli containing the endophytic alga were fixed for electron microscopy in 2% $KMnO_4$ and embedded in an Epon-Araldite mixture according to the methods of Mollenhauer (1959, 1964). Both thick (1μ) and thin sections of the specimens were cut on a Porter-Blum ultramicrotome. Thin sections were post-stained with 5% $Ba(MnO_4)_2$ to increase image contrast.

For light microscopy thalli containing endophytic algal colonies were fixed for 24 hr in formalin-acetic-acid, dehydrated, embedded in Tissuemat (mp 52.5°C), serially sectioned at 7μ, and placed on glass slides with Haupt's adhesive (Johansen, 1940; Jensen, 1962). Cytochemical procedures employed for localization studies were: the mercuric-bromphenol blue reaction (Mazia et al., 1953) for protein; the periodic acid-Schiff's reaction for insoluble carbohydrates (Hotchkiss, 1948); and ruthenium red to indicate the presence of pectic substances (Johansen, 1940).

RESULTS

The results of the experiments are summarized in Table 2. The control cultures were extremely chlorotic (Fig. 1) and only a few apices of the thalli remained green. In contrast, the experimental thalli were still green and growing vigorously (Fig. 2-4).

All but two of the *Nostoc* isolates entered into the thalli and produced typical endophytic colonies. No isolate of *Anabaena* formed endophytic colonies. However, usually both *Nostoc* and *Anabaena* isolates grew on the agar surface in intimate association with the thalli. In cases in which no entry of the algal isolate into the thalli was observed but in which a heavy growth of the alga occurred on the agar surface (Fig. 2), the thallus appeared almost normal. In cases in which the endophytic colonies developed within thalli (Fig. 3) without substantial growth of the alga on the surrounding agar surface, or cases in which both endophytic algal colonies were produced and substantial growth of the alga upon the substrate occurred (Fig. 4), little or no chlorosis of the thalli was observed. No hepatic species specificity was noted with respect to entry of the various *Nostoc* specificity was noted with respect to entry of the various *Nostoc* isolates used in

Table 2. Relation of *Nostoc* and *Anabaena* isolates and several *Anthoceros* and *Phaeoceros* species in culture.[a]

Algal strain	*P. laevis* (Tex.) E T A	*P. laevis* (Ala.) E T A	*A. husnoti* E T A	*A. punctatus* E T A
122	− P E	− P E	− P E	− P E
123	+ G E	+ G E	+ G E	+ G E
373[b]	− P Ec	− P Ec	− P E	− P E
388[b]	+ G E	+ G E	+ G E	+ G E
391[b]	+ G E	+ G E	+ P E	+ P E
393[b]	+ P E	+ P E	+ G E	+ G E
395[b]	+ G S	+ G S	+ G E	+ G E
396[b]	+ G E	+ G E	+ G E	+ G E
NMA	+ G S	+ G S	− P S	− P S
NMB	− P M	− P M	− P M	− P M
49	+ G E	+ G E	+ G E	+ G E
50	+ P S	+ P S	+ P S	+ P S
ND_1	− G Ec	− G Ec	− G Ec	− G Ec
ND_2	+ G S	+ G S	+ G E	+ G E
Control	− D −	− D −	− D −	− D −

[a] Readings were taken after 125 days. The letter designations on the above table are:
E = endophytic colonies formed (+ = present in the thalli, − = absent in the thalli.)
T = condition of the *Anthoceros* thallus (G = thallus green; P = thallus slightly chlorotic; D = only a few apices remaining alive.)
A = amount of blue-green algal growth upon agar surface (E = excellent; M = medium; S = slight; Ec = extremely heavy growth which covered portions of the thallus.)

[b] These algal isolates were obtained from endophytic colonies of other plants. See Table 1.

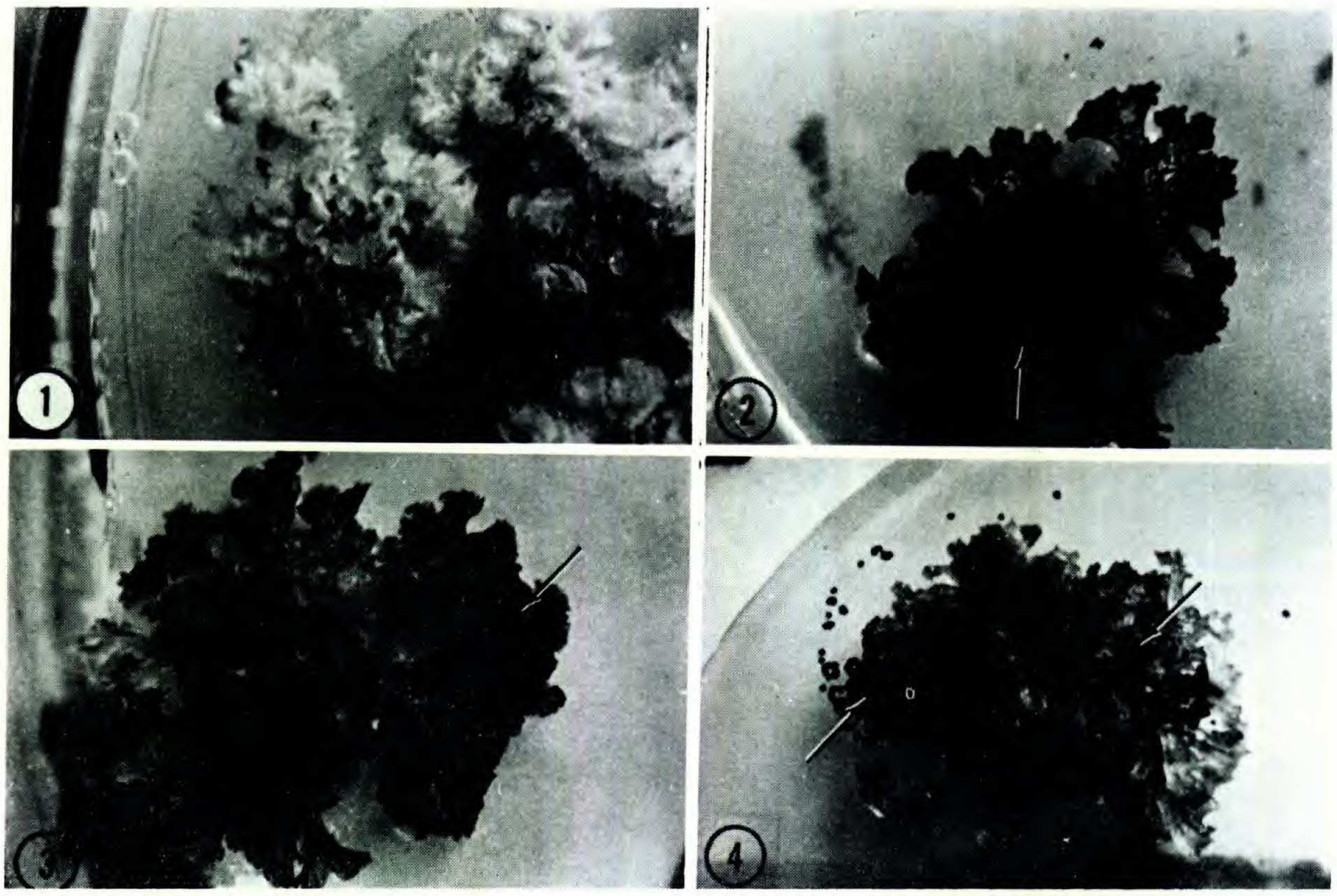


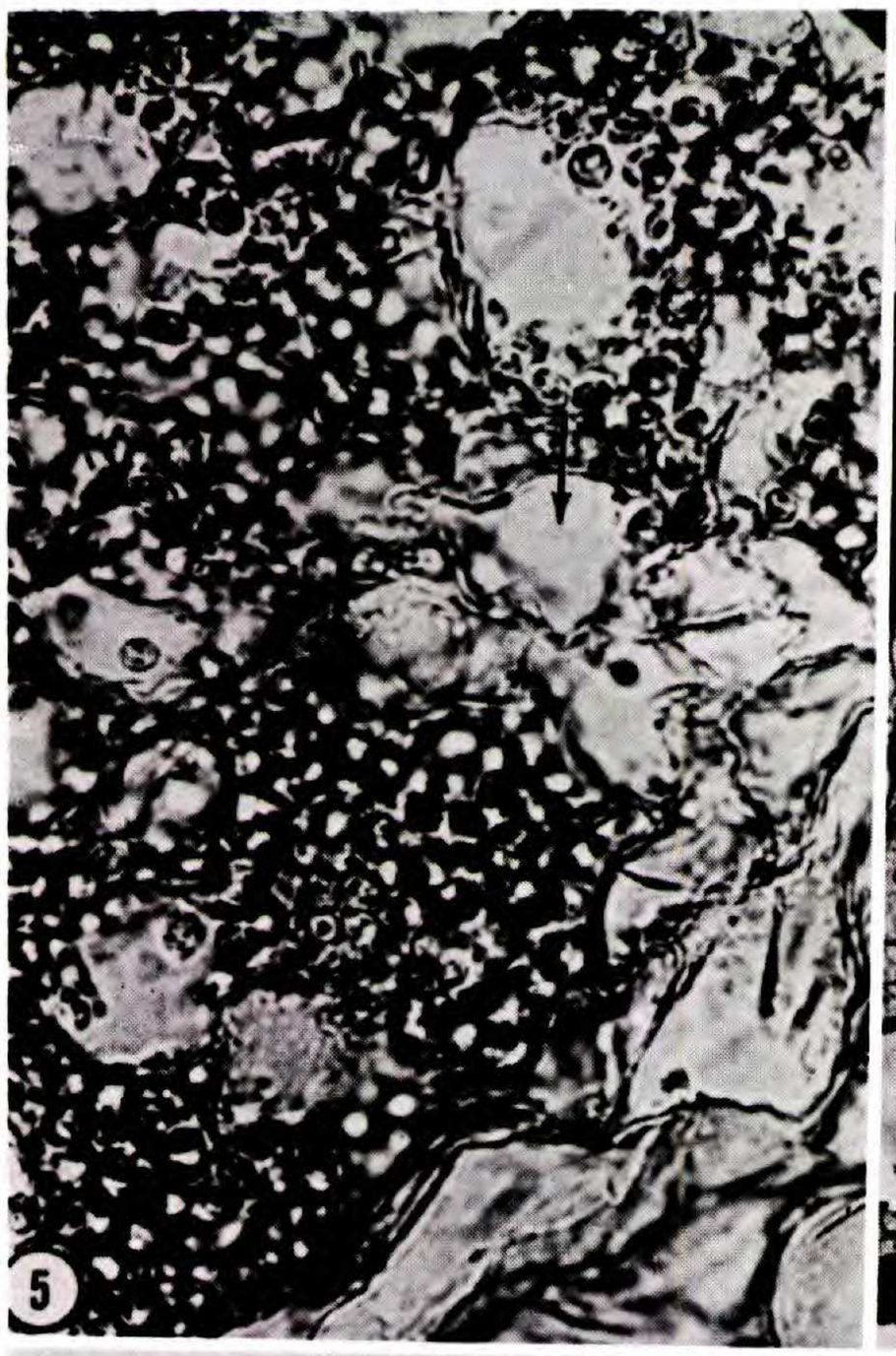
Fig. 1-4. Relation of *Anthoceros* to *Nostoc*. Fig. 1. *A. punctatus* 95 days after inoculation on nitrogen-deficient medium. Note the extremely chlorotic condition of the thallus, ×42. Fig. 2. *A. husnoti* which has been inoculated with *Nostoc* isolate ND_1. Note the absence of any endophytic colonies, but the presence of the heavy algal growth on the agar substrate (arrow), ×42. Fig. 3. *A. husnoti* which has been inoculated with *Nostoc* isolate NMA. Note the presence of endophytic algal colonies (arrow) but the absence of any algal substrate growth, ×42. Fig. 4. *A. husnoti* which has been inoculated with *Nostoc* isolate #49. Note the presence of endophytic algal colonies (arrow) and the presence of the algal growth upon the substrate, ×42.

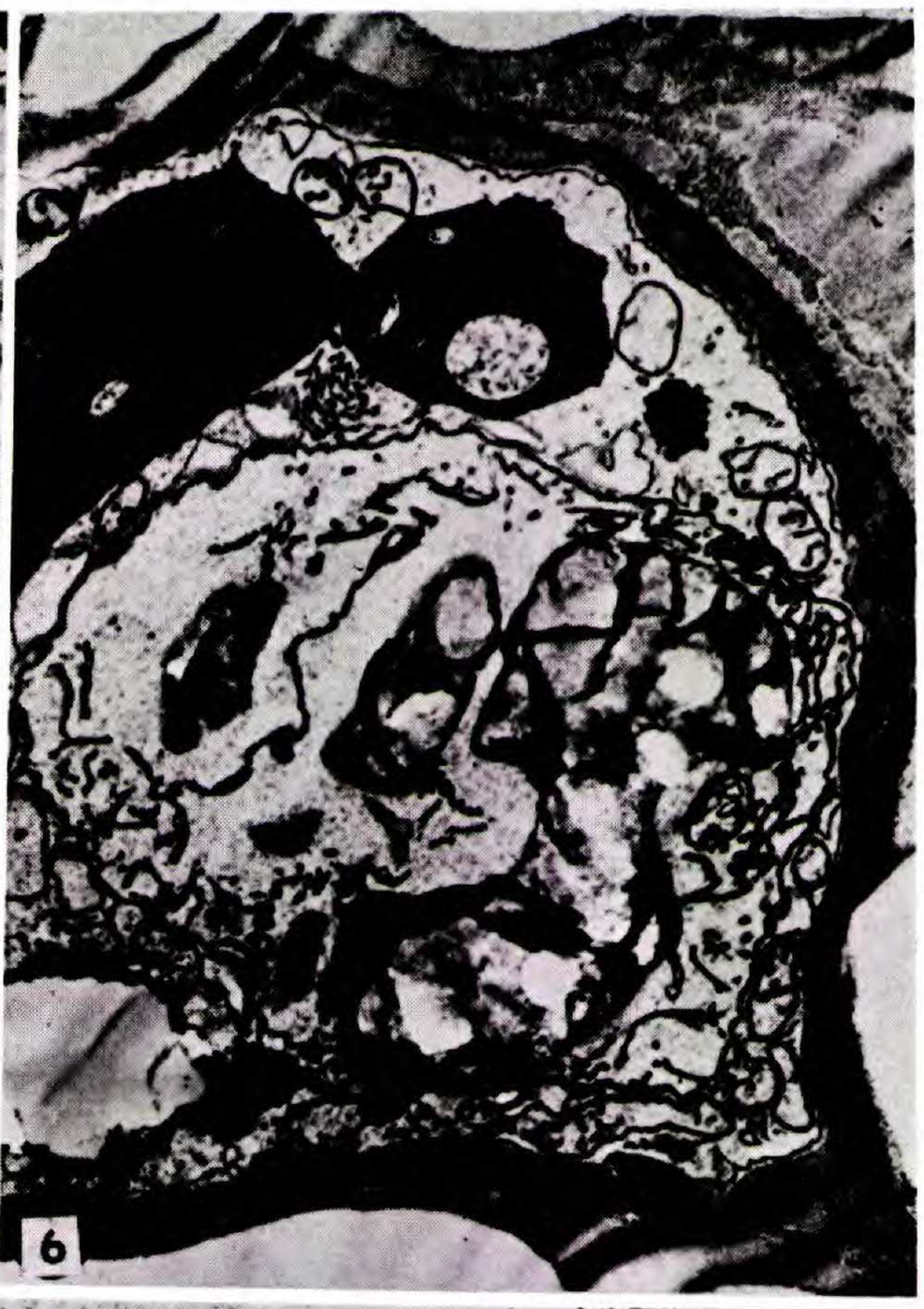
this experiment—e.g. if a particular algal isolate formed endophytic colonies in one species of the hornwort, all other species were also endophytized.

In cytochemical investigations of the endophytic relationship, the mucilaginous matrix of the thalloid cavity in which the blue-green algae were embedded was PAS-positive, indicating the presence of carbohydrates. This mucilaginous substance also stained pink with ruthenium red, a classical dye indicator for the presence of pectic substances. In other sections which were stained by the mercuric bromphenol blue reaction, only the blue-green algae stained positively for the presence of protein; no staining reaction occurred within the mucilaginous matrix of the liverwort.

Light microscopic examinations of 1μ sections of the infected thalli revealed basic morphological differences between cells of the mucilaginous cavity wall and other cells of the thallus. Cells adjacent to the cavity or which projected into it (Fig. 5) contained smaller and less developed chloroplasts and fewer vacuoles.

Ultrastructural studies supported the above observations (Fig. 6, 7). In sections of the "host" cells adjacent to the cavity or in sections of these cells within the cavity there were fewer and smaller electron dense vacuoles present; subcellular





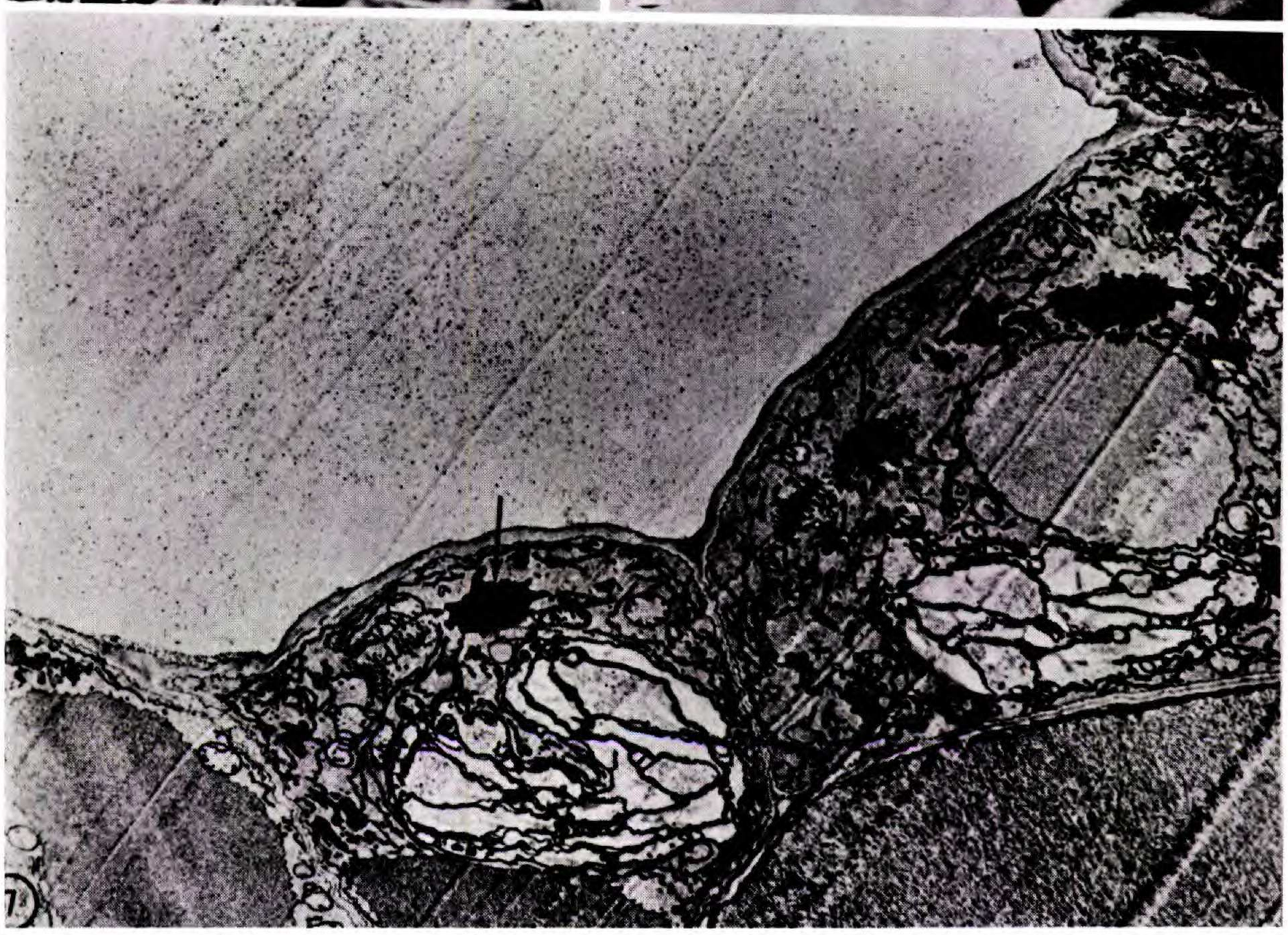


Fig. 5. Photomicrograph of a portion of a colony within the thallus of *Anthoceros punctatus*. Note the small densely staining cells adjacent to the cavity and transections (arrow) of the "host" tubular cells which project into the algal mass, ×800. Fig. 6. Electron micrograph of a portion of an *Anthoceros* "host" cell within an endophytic colony of *Nostoc*. Note the densely staining vacuole, the abundant mitochondria, elements of endoplasmic reticulum, and the plastid, ×14,400, $KMnO_4$ fixation. Fig. 7. Electron micrograph of a transection of the wall lining a developing mucilaginous cavity which has not yet been infected with *Nostoc*. Note the small cell size, the densely staining vacuoles (arrow), the numerous mitochondria, and elements of endoplasmic reticulum, ×7100, $KMnO_4$ fixation.

organelles were more numerous (possibly because of smaller cell size); and the plastids contained fewer internal elements as compared to those in thalloid cells not in contact with the cavity. Further ultrastructural studies utilizing the more recently developed aldehyde-fixation procedures may provide additional significant information with respect to the biotic relationship between the endophytic alga and its host. The algal cells generally were poorly fixed by the ultrastructural techniques employed. Further studies which are currently underway should provide further data with respect to the biotic relationship at the ultrastructural level.

Discussion

In each instance in which the algal isolate entered into the thalli and produced typical globose colonies and/or grew upon the substrate in intimate contact with the thalli, a significant difference in chlorosis between the experimental and control cultures was observed. The absence of the chlorotic conditions within the experimental plants indirectly suggests the ability of the blue-green algal isolates to fix atmospheric nitrogen and to provide an available source of nitrogen to the liverworts used in this experiment. These data do not support the conclusions of Pierce (1906). Additional studies for direct evidence of utilization of alga-fixed nitrogen are planned. In these experiments N^{15} will be used and the cultures will be grown in similar fashion with proper controls. Analyses for the presence of N^{15} within the thalli should provide direct evidence to support the above experiments.

Cytochemical and ultrastructural observations suggest that the biotic coaction between the liverwort and the blue-green alga may be also beneficial to the latter if the alga is capable of catabolizing the carbohydrate components of the mucilaginous matrix. In addition, Cobb & Meyers (1964) reported that in the blue-green alga *Anabaena cylindrica* nitrogen fixation is depressed by light intensities greater than those required to saturate photosynthesis. Their data show increased nitrogen fixation by the blue-green alga under light limiting conditions. That light intensity within the endophytic colony might be a limiting factor for photosynthesis and other photochemical events is suggested, firstly, by the lesser development of chloroplasts of thalloid cells which protrude through the algal mass, and, secondly, by the location of the cavity within the thallus as the cavity usually is covered by several layers of thalloid cells. Hence, if light is indeed limiting, nitrogen fixation by the endophytic alga would be enhanced.

Additional studies are needed to provide direct evidence of utilization of algal-fixed nitrogen, to define or isolate the algal influence which causes increased growth in cells surrounding the mucilaginous cavity, and to investigate the ability of the blue-green alga to utilize the carbohydrates of the cavity as a carbon energy source for metabolic activities under light limiting conditions. The present studies have shown that the biotic relationship between the blue-green alga and its liverwort host is, if not protocooperative, certainly beneficial to the hornwort host.

Literature Cited

Bischoff, H. W. & H. C. Bold. 1963. Phycological studies IV. Some soil algae from Enchanted Rock and related algal species. Univ. Texas Publ. #6318.

Bond, G. & G. D. Scott. 1955. An examination of some symbiotic systems for fixation of nitrogen. Ann. Bot. **19:** 67-77.

Campbell, D. H. 1895. The structure and development of mosses and ferns. The MacMillan Company, New York.

Cobb, H. & J. Meyers. 1964. Comparative studies of nitrogen fixation and photosynthesis in *Anabaena cylandrica*. Amer. Jour. Bot. **51:** 753-762.

Hofmeister, W. 1862. The higher cryptogamia. Ray Society.

Hotchkiss, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide in fixed tissue preparations. Arch. Biochem. **16:** 131-141.

Jensen, W. A. 1962. Botanical Histochemistry: Principles and Practice. W. H. Freeman & Company, San Francisco.

Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill Book Company, New York.

Kelly, A. G. & S. N. Postelthwait. 1962. Effect of 2-chloroethyltrimethylammonium chloride chloride on fern gametophytes. Amer. Jour. Bot. **49:** 778-786.

Leitgeb, H. 1878. Die Nostoccolonien im Thallus der Antocerotees. Sitzungsber. K. Akad. Wiss. in Wien **77:** 79-95.

Mazia, D., P. A. Brewer & M. Alfert. 1953. The cytochemical staining and measurement of protein with mercuric bromphenol blue. Biol. Bull. **104:** 57-67.

Mollenhauer, H. H. 1959. Permanganate fixation of plant cells. Jour. Biochem. Biophy. Cytol. **6:** 431-435.

———. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Tech. **39:** 111-114.

Näf, U. 1962. Developmental physiology of lower archegoniates. Ann. Rev. Plant Physiol. **13:** 507-532.

Parihar, S. N. 1961. An introduction to *Embryophyta*, Volume I: *Bryophyta*. Central Book Depot. Allahabad, India.

Pierce, G. J. 1966. *Anthoceros* and its *Nostoc* colonies. Bot. Gaz. **42:** 55-58.

Watanabe, A. & T. Kiyohara. 1963. Symbiotic blue-green algae of lichens, liverworts, and cycads. 189-196. Microalgae & Photosynthetic Bacteria. Japan Publ. Trading Co., Tokyo.

Winter, G. 1935. Über die Assimilation des Luftstickstoffs durch endophytische Blaualgen. Beitr. Biol. Pfl. **23:** 295-335.